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Enterocyte differentiation is compatible with SV40 large T expression and loss of p53 function in human colonic Caco-2 cells

Status of the pRb1 and pRb2 tumor suppressor gene products

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Abstract Transfer of the *SV40 large-T* (LT) oncogene into isolated human and murine intestinal epithelial cells induced alterations of the ultrastructural organization and polarization of the resulting immortalized cell lines. We now demonstrate that the functional expression of the SV40 LT antigen in Caco-2 cells did not alter phenotypic markers of differentiation, including expression of villin, sucrase–isomaltase, brush border and dome formation. As compared to parental cells, the transfected Caco-2 LT9 cells exhibited similar growth curves and no invasive properties in vitro. The major oncogenic function of the SV40 LT antigen in transfected Caco-2 cells is associated with reduced latency times necessary for the manifestation of tumors in athymic nude mice. The Caco-2 cell line contained deleted and mutant p53 alleles (stop codon in position 204) and has no detectable truncated p53 protein by Western blot. Molecular complexes between the SV40 LT antigen and the retinoblastoma-related proteins pRb1 and Rb2 were clearly identified at the different phases of the growth curve. When compared to normal human colonic crypts, Caco-2 cell differentiation is related to partial redistribution of pRb1 into hypophosphorylated, anti-proliferative forms. The pRb2 protein is found elevated in a subset of human colorectal tumors and their corresponding liver metastases. We conclude that: (1) Caco-2 cells exert a dominant control against the oncogenic functions of the LT antigen; (2) loss of p53 function is not restrictive for the establishment of polarity and differentiation of the enterocyte lineage; (3) the levels and phosphorylation status of the Rb1 and Rb2 proteins may play important roles in the proliferation and differentiation of normal and neoplastic human colonic mucosa.

1. Introduction

The cellular and molecular mechanisms of tumor progression involve point mutations, chromosomal rearrangements and loss of tumor-suppressor genes [1–4]. Transfer of genetic elements derived from mammalian DNA tumor viruses is often associated with the development of cellular proliferation, immortalization and transformation of mesenchymal and epithelial cells [5,6]. Genetic and biochemical studies have shown that the SV40 large T antigen (SV40 LT) is composed of several functional domains interacting with different cellular targets in the cytoplasm and nucleus [5–7]. For example, the SV40 LT antigen is known to form complexes with the tumor-suppressor gene products p53 and p105Rb1, and to antagonize their functions regarding proliferation, differentiation and senescence. In this connection, we have previously demonstrated that the viral oncogenes large T of SV40 and polyoma, the early region E1A of adenovirus 2 extend the life span of primary epithelial cells isolated from the gastrointestinal tract [8–13]. Transfection of gastric and intestinal epithelial cells by plasmid vectors and retroviruses carrying the *SV40 LT* oncogene has resulted in the establishment of permanent cell lines at various stages of the neoplastic transformation. In addition, we have investigated the consequences of the cellular immortalization and transformation upon some differentiation criteria, including cell polarity and ultrastructural characterization, expression of specific markers of the human and murine digestive epithelia. We have found that the introduction and expression of SV40 LT in primary gastric and intestinal epithelial cells is correlated with altered differentiation of the corresponding cell lines.

In the present study, we examined the effects of SV40 LT on the growth and differentiation of the human colonic adenocarcinoma Caco-2 cells. This cell line provides an appropriate model to study the neoplastic progression and differentiation in intestine since Caco-2 cells are poorly tumorigenic in nude mice and grow as typical enterocytes in culture [14]. The status of the tumor suppressor proteins p53, Rb1 and Rb2 was also characterized in the parental and SV40 LT-transfected Caco-2 cell lines.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham Corp. (Les Ullis, France). The anti-Rb1 antibodies C36 (mouse mAb for immunoprecipitations) and C15 (rabbit pAb for Western blots) were from Santa Cruz Biotechnology (Tebu, Le Perray and Yvelines, France). The affinity-purified rabbit anti-Rb2 pAb was directed against the C-terminal region of the protein. The mouse mAb to SV40 LT antigen (Pab 101), the p53 mouse mAb DO-1 specific of the amino terminal region 37–45 of the p53 protein, and the mouse mAb 421 specific of the carboxyl terminal region of p53 (371–380 C-terminal region) were from Oncogene Science (Paris, France). The mouse mAb PAb762 directed against the Py antigens was kindly provided by Dr. S. Dilworth (Hammersmith Hospital, London, UK). The expression vector pMSPE encoding the SV40 LT antigen and neoresistance was a generous gift from Dr. M. Schuermann (IMT, Philipps-Universität Marburg D-3550, Germany). All other chemicals were of analytical grade.

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2.2. Cell culture and transfection

The human colonic adenocarcinoma Caco-2 and HT-29 cell lines were obtained from Dr. A. Zweibaum (INSERM U 178, Villejuif, France). The CFI-3 cell line was established after transfection of primary human intestinal cells by the *SV40 LT* oncogene [9]. In standard conditions, all three cell lines were grown at 37°C in Dulbecco's modified Eagle medium (DMEM, from Gibco BRL, Grand Island, NY) supplemented with 8 mM L-glutamine, antibiotics, and 15% fetal calf serum (Deutscher, France) in an atmosphere of 95% air/5% CO_2. Cells were passaged weekly, using 0.005% (w/v) trypsin, 0.02% (w/v) EDTA in a 1:10 split ratio. The human colonic adenomatous cell line PC AA/C1 cell line [15], designated as the PC cells throughout this study, and their polyoma large T-transfected counterparts PCPy/csrc cells, were cultured in the same culture medium supplemented with 0.2 U/ml insulin and 1 μg/ml hydrocortisone.

Caco-2 cells at passage 67 were harvested and washed in a solution containing 10 mM Na_2HPO_4/NaH_2PO_4, 250 mM sucrose and 1 mM $MgCl_2$ (pH 7.45). About 10^7 Caco-2 cells were incubated for 10 min at 4°C in 1 ml of the same buffer, in the presence of 10 mg of the pMSPE vector recombined with the origin-defective mutant of SV40. The Caco-2 cells were transiently permeabilized by a BioRad apparatus (675 V/cm, 25 μFd). Transfected cells were allowed to grow for 2 days, after which the selection medium containing 1 mg/ml of the neomycin analogue G418 was added to the cultures. Dishes were then left for 2 weeks with weekly selection medium change. The surviving colonies were isolated using cloning cylinders, amplified and analyzed for the expression of the viral transgene. The different clones of Caco-2 cells isolated after transfer of the SV40 LT and neogenes were designated by the suffix (-LT).

2.3. Tissue samples

Tissue samples obtained from patients at surgery (Centre de Chirurgie Digestive, Service du Pr. Parc, Hôpital Saint-Antoine) were immediately frozen in liquid nitrogen. Specimen from non-necrotic malignant tissue and adjacent non-neoplastic mucosa were dissected, snap-frozen in liquid nitrogen and stored at −80°C until protein extraction. The specimens included adenocarcinomas of Dukes' stages B, C and D, and liver metastasis.

2.4. RNA isolation and Northern blot

Total RNAs were isolated by guanidinium isothiocyanate extraction and cesium chloride density gradient ultracentrifugation. After denaturation, RNA samples underwent electrophoresis in 1% agarose, 2.2 M formaldehyde gels, were transferred onto Hybond N+ nylon membranes (Amersham, UK), and hybridized with the ^{32}P-labeled probe (Megaprime, Amersham). The membranes were then washed twice for 15 min at room temperature in 2×SSC, 0.1% SDS (2×SSC is 0.3 M NaCl, 30 mM sodium citrate, pH 7.0) and incubated for 60 min at 42°C in 0.1×SSC, 0.1% SDS. Autoradiographs were performed at −70°C using Kodak X-Omat AR films (Rochester, NY) and a Chronex Quanta III intensifying screen (Dupont). The relative abundance of RNA per lane was judged to be similar by comparing the ethidium bromide or methylene blue staining of the ribosomal bands.

The SV40 LT probe consisted of the 5.2-kb *Bam*HI fragment of the pSV40 plasmid containing the viral sequence without the origin of its replication [9]. The RB probe was the human cDNA isolated from the pCMV-HRB plasmid, a generous gift from Dr. R. Weinberg (Cambridge, MA). The PCV 108 cosmid containing a *Cla*I–*Eco*RI fragment of the *c-myc* gene (third exon) was used as a probe [16]. The villin probe was the human cDNA isolated from the plasmid pSP64V19 after digestion by *Bam*H1 and *Sal*1 (Dr. E. Pringault, Paris, France).

2.5. P53 gene analysis

Since the vast majority of the mutations in the *P53* gene are located in exons 5–9, this region was analyzed by PCR and direct sequencing of the amplified products. Genomic DNA (150 ng) was amplified in 100 μl of reaction mixture containing 0.5 μM of each primer, 0.1 mM of each dNTP and 5 units of Taq polymerase (Perkin-Elmer Cetus), in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 2.5 mM magnesium chloride and 0.001% gelatin.

Primers were synthesized by the phosphoramidite method with a 391 DNA synthesizer (Applied Biosystems). Names and sequences were as follows:

E5	sense	5′TTCAACTCTGTCTCCTTCCT
	antisense	5′CAGCCCTGTCGTCTCTCCAG
E6	sense	5′GCCTCTGATTCCTCACTGAT
	antisense	5′TTAACCCCTCCTCCCAGAGA
E7	sense	5′GTAAAGCTTGTTGGCTCTGACTG-TACCACC
	antisense	5′ACAAAGCTTCCTGGAGTCTTC-CAGTGTGAT
E8	sense	5′TTCCTTACTGCCTCTTGCTT
	antisense	5′AGGCATAACTGCACCCTTGG
E9	sense	5′TTGCCTCTTTCCTAGCACTG
	antisense	5′CCCAAGACTTAGTACCTGAA

Reactions were performed for 35 cycles comprising a denaturation step (30 s at 94°C), followed by primer annealing (15 s, 55°C) and elongation for 30 s at 72°C. Double-strand amplified DNA was purified from the remaining primers by centrifugation through Centricon-100 columns (Amicon), according to the manufacturer's instructions. Amplification products were controlled and quantified by gel electrophoresis on 2% agarose gels. The PCR products (100 ng amplimeres) were subjected to a dideoxy-termination sequencing procedure using the Prism M reaction Kit, according to the protocol supplied by Applied Biosystem. Taq sequencing reactions were carried out for 25 cycles in a thermal cycler Beckman 9600, for 30 s at 96°C and 4 min at 60°C. Extended fragments were purified from uncorporated nucleotides and primers through quick spin columns (Boehringer). The reaction products were dried, resuspended in 4 μl of deionized formamide containing 50 mM EDTA (pH 8), heated to 90°C for 2 min, transferred to ice and loaded on a 6% polyacrylamide, 8 M urea gel. Gels were run for 12 h on ABI Model 373 A automated DNA sequencer. The chain reactions were performed on genomic DNA samples prepared from cultured Caco-2 cells at three different passages.

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out on total RNA (1 μg) extracted from cultured Caco-2 and HT-29 cells or human tissue resections for colorectal cancer. RNA was reverse transcribed in 20 μl of final volume containing 500 μM dNTP, 10 mM DTT, 0.5 U/μl RNasin (Promega Corporation), 5 μM random hexamers and 10 μg/ml reverse transcriptase (Gibco BRL, Bethesda, MD). The RT reactions were terminated by heating at 96°C for 5 min and quick-chilled on ice. The corresponding cDNA (5 μl) was added to 25 μl of the PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM $MgCl_2$, 0.001% gelatin, 0.05 U Taq DNA polymerase (Beckman, USA) and 0.5 mM of two sets of primers corresponding to p53 and β-actin sequences. The PCR reaction was performed in a Perkin-Elmer 9600 thermocycler (Cetus). Each cycle consisted of 15 s denaturation at 94°C, 15 s annealing at 58°C and 30 s extension at 72°C. Negative controls were done using RNA samples processed in the absence of reverse transcriptase. PCR products (10 μl) were then electrophoresed in 2% agarose gels. Fluorescence intensity of the PCR products was automatically measured using the Genescan Software in the ABI model 373 spectrofluorimeter (Applied Biosystem). The optimal number of PCR reactions to allow quantification of the expression of the *p53* and β-*actin* genes was 26 cycles. The amount of the p53 mRNA signal was expressed as arbitrary densitometric units (AU) and normalized for the level of mRNA encoding β-actin.

2.6. Western blot, immunoprecipitation, and immunofluorescence

The parental and SV40 LT-transfected Caco-2 cells (5×10^6 cells) were solubilized in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 8), 120 mM NaCl, 0.5% NP40 (v/v) and aprotinin (10 μg/ml). Denaturation was performed for 2 min in the sample buffer containing 50 mM Tris-HCl (pH 6.5), 1% SDS, 10% glycerol, 20% β-mercaptoethanol and 0.002 bromophenol blue. Samples were adjusted to a protein concentration of 100 μg/well and submitted to electrophoresis in a 7% polyacrylamide slab gel. Proteins were transferred onto Hybond-C-Extra nitrocellulose membranes (Amersham) at 0.8 mA/cm^2 for 1 h, using a Semidry transfect unit Multiphor II (LKB Bromma, Sweden). The membranes were briefly stained with Ponceau S to assess the equality of protein transfer and blocked overnight with 5% skimmed milk powder in Tris-saline buffer, containing 10 mM Tris-HCl, 150 mM NaCl (pH 8). Membranes were then incubated for 1 h at 22°C, with a 1:100 dilution of either the mouse anti-SV40 LT anti-

gen mAb (Pab 101), the Rb1 pAb C15 or the p53 mAbs against the N- or C-terminal regions of the protein.

The immunoblots were then washed in Tris-saline buffer containing 0.05% Tween-20, incubated for 1 h at 22°C with a 1:1000 dilution of the corresponding secondary polyclonal sheep antibodies conjugated with horse radish peroxidase, and probed using the enhanced chemiluminescence system (ECL, Amersham, UK).

For immunoprecipitation of the pRb1 and Rb2 proteins, human intestinal cells in culture were homogenized and lysed in ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM EDTA, 0.5% NP40, 1 mM sodium orthovanadate, 50 mM NaF, 45 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonylfluoride. Cell lysates were cleared by centrifugation at 15000×*g* for 10 min and the supernatant containing 2 mg cell protein was incubated for 1 h at 4°C with either the C36 RB1 (1 μg protein antibody) or the pRb2 antibody (5 μl) directed against the COOH-terminus of the protein. Then, 40 ml of protein G-Sepharose was added and the immunoprecipitation was performed for 1 h at 4°C. The immunoprecipitates were washed 3 times in 1 ml of the same buffer, resuspended in sample buffer and boiled. Electrophoresis was performed in the conditions described above.

Intestinal cells grown on Labtek (PolyLabo, France) were fixed for 10 min at 4°C in a 2:1 mixture of methanol/acetone. This material was incubated for 90 min with a 1:10 dilution of the primary mAb against the SV40 LT antigen and then with the anti-mouse IgG coupled to fluorescein as second antibody (Silenus, France). Preparations were observed with a microscope equipped with epifluorescence optics (Leitz).

2.7. Cell proliferation and tumorigenicity

Parental and transfected Caco-2 cells were seeded at the initial density of 50000–100000 cells per 60 mm Falcon dishes in the standard culture medium containing either 15, 5 or 1% fetal calf serum. In some experiments, cell proliferation was measured in the absence of serum or glutamine. The culture medium was renewed every 48 h and the cell count was performed daily, using a Coulter Counter (Coultronics, Luton, UK).

About 10^7 Caco-2 cells (parental or transfected cell lines) were injected subcutaneously into athymic nude mice, 4–6 weeks old. All animal were monitored weekly for the presence and size of the tumors over a period of 3 months. The latent period to tumor development was defined as the period until a hard palpable and measurable tumor appeared. Tumor growth was evaluated by 3-dimensional calliper rule measurements of length (*L*), width (*W*) and height (*H*). Tumor volume was calculated by the formula: $A = 4/3\pi \times L \times W \times H$. Tumors were removed when they reached about 8 mm in diameter to minimize discomfort to the mice.

2.8. In vitro invasion assay

The invasive potential of the parental and SV40 LT-transfected Caco-2 cells was investigated in vitro, in type I collagen gel and chick heart fragment invasion assay, as previously described [17,18]. The invasion index was expressed as the percentage of cells invading the gel over the total number of cells. An invasion index higher than 10% designated highly invasive cells.

2.9. Ultrastructural analysis and sucrase activity

Caco-2 cells were cultured for 10 days after the acquisition of confluence, and fixed for 2 h at room temperature in Cacodylate buffer (pH 7.2) containing 2% formaldehyde, 4% glutaraldehyde and 0.06% picric acid. After postfixation in osmium tetroxide, the samples were processed for electron microscopy, as previously described [18]. Sucrase activity was measured according to Dahlqvist, using sucrose as substrate [18].

3. Results

3.1. Transfection of Caco-2 cells by the SV40 LT oncogene

Parental Caco-2 cells at passage 67 underwent electropermeabilization and were transfected by the pMSPE expression vector encoding the SV40 LT antigen and neoresistance. We observed that this expression vector pMSPE is efficient to immortalize human thymic epithelial cells in primary culture

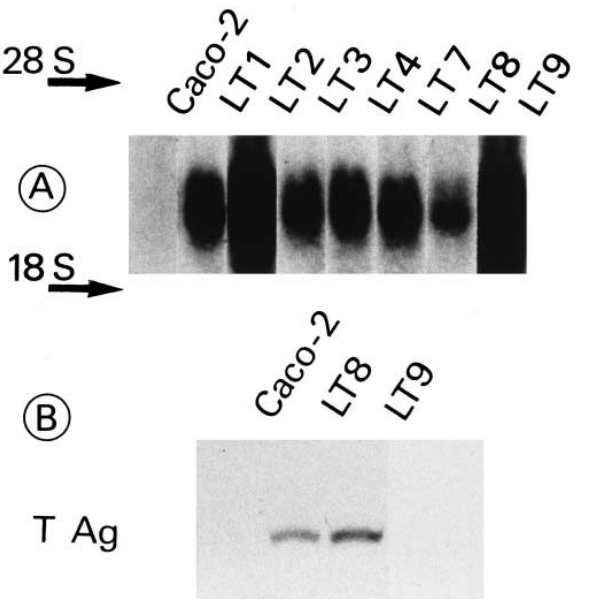

Fig. 1. *SV40 LT* oncogene expression in SV40 LT-transfected Caco-2 cells. A: Northern blot analysis of the SV40 LT transcript. Total RNA samples were extracted from the parental Caco-2 cell line and nine different clones obtained after selection of the transfected cells using 1 mg/ml G418. The G418-resistant colonies were designated Caco-2 LT1 to -LT9. B: Western blot analysis of the SV40 LT oncoprotein. Cellular extracts were prepared from parental and transfected Caco-2 LT8 and -LT9 cells, after selection by G418. T Ag: SV40 LT antigen.

(C.G., unpublished observations). The Caco-2 cells expressing the transgene sequences were selected in culture medium containing 1 mg/ml of the neomycin analogue G418. The non-transfected parental cell line treated with 1 mg/ml G418 did not survive at this drug concentration. After selection by G418 for 2 weeks, nine clones were obtained and subsequently analyzed for the accumulation of the SV40 LT transcripts (Fig. 1A). Northern blot analysis revealed that the LT2 and LT9 clones exhibited high accumulation of the SV40 LT mRNA identified as a main band of 2.5 kb. In contrast, the LT8 subclone showed lower abundance of the SV40 LT transcripts, while no hybridization occurred using the RNA extracted from the LT5- and LT6-resistant colonies (data not shown). The persistence of the transgene expression was checked in Caco-2 LT9 cells at passages 71 and 81. The Caco-2 LT9 clone expressed moderately higher levels of T antigen (T Ag) than the -LT8 clone (Fig. 1B).

Approximately 80% of the Caco-2 LT9 cells expressed the SV40 LT antigen within their nuclei by immunofluorescent staining. This expression remained stable over passages in culture (data not shown). Similar nuclear staining was observed in the SV40 LT-immortalized rat intestinal cell line SLC-44 [8].

3.2. Proliferation, tumorigenicity and invasiveness

Growth of parental and SV40 LT-transfected Caco-2 cells was measured by seeding equal numbers of cells (0.5 to 1×10^5 cells) into 60 mm Petri dishes. Cell number was determined at different times, during the exponential phase of growth and after the acquisition of confluence.

The growth curves of the Caco-2 and -LT9 cell lines were similarly affected by decreasing the serum concentrations from

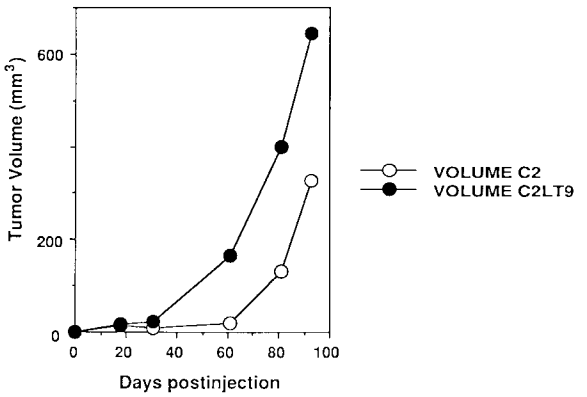

Fig. 2. Tumorigenicity of the parental and Caco-2 LT9 cells. Nude mice were inoculated subcutaneously with 10^7 cells. Each point is the mean of 20 measurements representative of four separate experiments. Caco-2 (○) and Caco-2 LT9 cells (●).

15% to 1% in the culture medium. The parental and transfected Caco-2 cells also grew at similar rates in the absence of serum or glutamine, and were propagated for 11–15 passages under these restrictive culture conditions (data not shown). Glutamine was described as an essential nutriment for the growth of intestinal epithelial cells [21–23]. Accordingly, the doubling time increased from 32 h in Caco-2 and -LT9 cells cultured in the presence of serum, to 49 h in Caco-2 cells deprived of serum or glutamine.

Following the introduction into nude mice of 10^7 cells, the parental Caco-2 cell line grew slowly to produce significant tumor formations by 8–11 weeks following injection (Fig. 2). In contrast, expression of the SV40 LT antigen in the Caco-2 LT9 cell line leads to shorter latency times so that tumors were observed by 4–8 weeks with similar growth rates once initiated.

The invasive properties of the parental and SV40 LT-transfected Caco-2 cells were evaluated by measuring cellular invasion of type I collagen gel and embryonic chick heart fragments. As shown in Fig. 3, expression of SV40 LT in Caco-2 cells is not associated with the acquisition of invasiveness in the collagen gels assay. Similarly, Caco-2 and -LT9 cells did not invade into the chick heart (data not shown). They formed epithelioid monolayers and multilayers surrounding the heart fragments. Fibroblastic transformation of the cardiac muscle was frequently observed at the vicinity of the Caco-2 cells.

3.3. *Morphological and functional differentiation*

Post-confluent cultures of Caco-2 cells were characterized by the progressive emergence of domes, as previously described [14]. Expression of the SV40 LT antigen in Caco-2 LT8 and -LT9 cells was also associated with dome formation with similar size and density.

Parental and transfected Caco-2 cells cultured in the standard culture medium had the characteristic ultrastructural morphology of differentiated enterocytes. They formed a continuous monolayer of polarized cells displaying an apical surface covered with abundant microvilli (data not shown). Central actin microfilaments in the microvillus core extended deeply into the cytoplasm. Apical junctional complexes, including tight junctions and desmosomes have often been observed in both cell lines. Sucrase-isomaltase activity was similar in Caco-2 and Caco-2 LT9 cell line during the exponential phase of growth and confluent monolayers (data not shown). This activity gradually increased 10 days after confluence and this evolution was not prevented in Caco-2 cells transfected by the

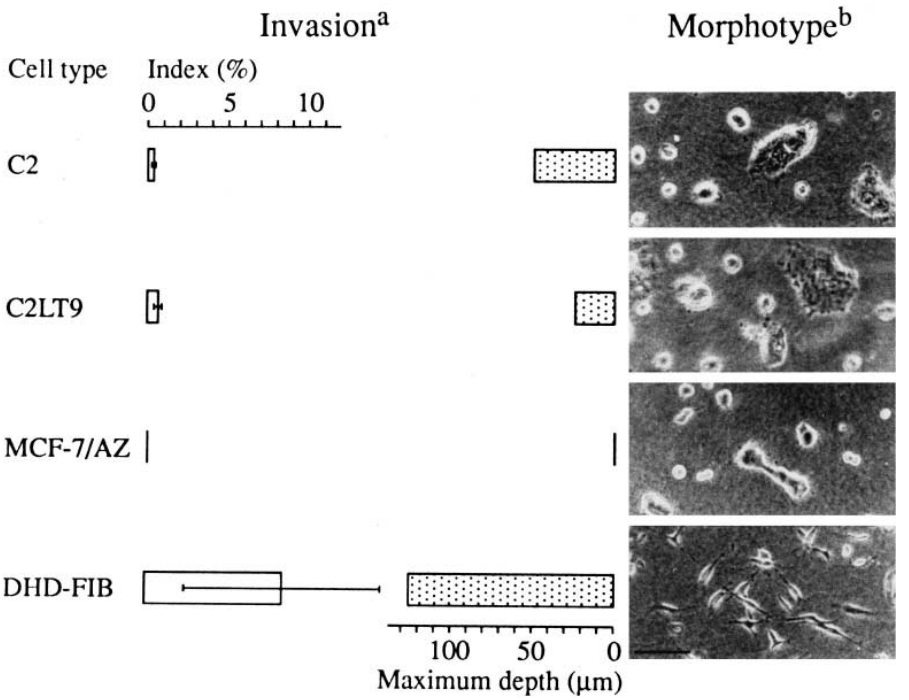

Fig. 3. Invasive potential of the parental and Caco-2 LT9 cells. a: The invasion index was expressed as the percentage of cells invading the gel over the total number of cells. b: Maximum depth of cell penetration into the gel. MCF-7/AZ cells are non-invasive variants selected from the parental human mammary cell line MCF-7. DHD-FIB cells belong to a myofibroblast cell line derived from a chemically induced rat colon cancer. These two cell lines represent non-invasive and invasive controls in this assay. C2 and C2 LT9: Caco-2 and Caco-2 LT9 cells.

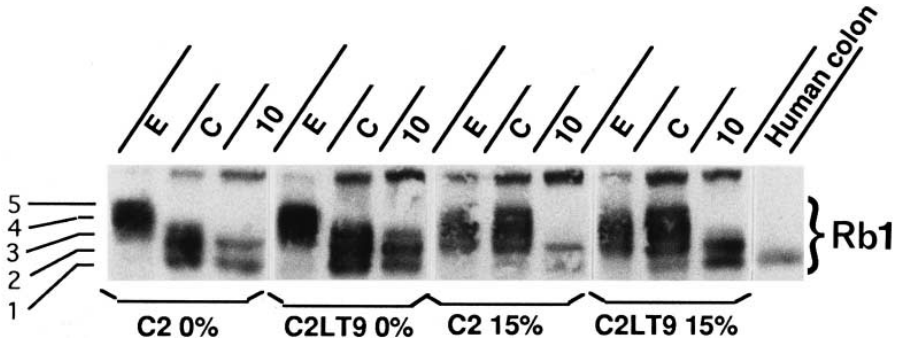

Fig. 4. Expression and phosphorylation pattern of pRb1 during enterocyte differentiation of parental and Caco-2 LT9 cells. The amount and the phosphorylation status of pRb1-immunoreactivity was analysed by Western blotting in parental Caco-2 cells (C2) and Caco-2 LT9 cells (C2 LT9) cultured in the presence of SVF (15%) or in serum-free medium (0%). Protein extracts were harvested in Caco-2 cells during the exponential phase of growth (E), at confluence (C) and in 10 days post-confluent monolayers (10). The five bands shown represent forms of pRb1 with different degrees of phosphorylation. Data are representative of three other experiments.

SV40 LT oncogene. Similarly, the two specific transcripts of the *villin* gene (2.7 and 3.5 kb) were equally accumulated in the two cell lines during the three phases of the cell culture considered (data not shown).

3.4. *Phosphorylation pattern of pRb1 and enterocyte differentiation*

The accumulation and the pattern of phosphorylation of pRb1 was analyzed in Caco-2 and -LT9 cells at various stages of growth and differentiation (Fig. 4). Five different phosphorylated forms of pRb1 protein were clearly detected as closely spaced bands at approximately 105–110 kDa. In standard culture conditions (15% SVF) or in the absence of SVF (0%), the majority of pRb1 was hyperphosphorylated during the exponential growth and at confluence. In contrast, pRb1 exhibits a hypophosphorylated pattern in terminally differentiated enterocytes obtained 10 days after confluence. In comparison, only one hypophosphorylated form of pRb1 could be detected in freshly isolated human colonic crypts. Expression of the SV40 LT antigen in Caco-2 LT9 cells resulted in accumulation and persistence of two hyperphosphorylated forms in bands 2 and 3.

3.5. *pRb1/SV40 LT interactions and p53 status*

As shown in Fig. 5, the Rb1/p105 protein interacts with the large T antigen in transfected Caco-2 LT9 cells, as demonstrated by immunoprecipitation of pRb1 with the pAb C36 and subsequent revelation of the SV40 LT antigen by Western blot. The pRb1–LT antigen complex is clearly identified in transfected cells harvested during the exponential phase of growth, at confluence or in 10 days post-confluent monolayers.

In contrast, Western immunoblot analysis indicated that the Caco-2 cell extracts do not contain detectable levels of p53 protein (Fig. 5). The same situation was observed in SLC-44 cells, freshly isolated human colonic crypts, and K-562 cells which have no p53 mRNA. In contrast, human colonic HT-29 cells accumulated high levels of immunoreactive p53 protein by Western blot, using the mAbs DO-1 and 421 that recognize respectively the amino-terminal transactivation domain of p53 (N-t) and the carboxy-terminal region (C-t). This cell line harbours a G/A (Arg > His) mutation in codon 273 of the *p53* gene [19]. Since the wild-type p53 protein has a short half-life of few minutes and is not detectable immunochemi-

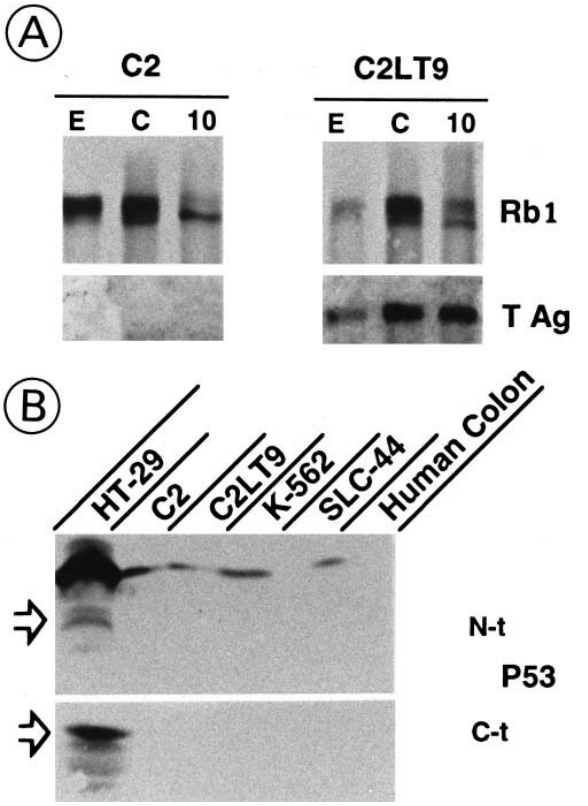

Fig. 5. Detection of the pRb1–SV40 LT antigen complexes. A: Immunoblot analysis of the pRb1 and SV40 LT in pRb1 immunoprecipitates prepared from parental (C2) and SV40 LT-transfected Caco-2 cells (C2 LT9). pRb was first immunoprecipitated using the C36 pAb and revealed by Western blot using the mAb C15. The nitrocellulose membrane was then stripped of bound antibodies and reprobed with the mouse mAb to SV40 LT antigen (T Ag). E: exponential phase of growth; C: confluent monolayers; 10: 10 days post-confluent Caco-2 cells. B: Western blot analysis of the p53 protein in parental (C2) and transfected Caco-2 LT9 cells (C2 LT9); HT-29 human colonic cells as a positive control, K562 human myelogenous leukemia cells as a negative control, SLC-44 cells and human colonic epithelial cells (HC). The p53 protein was revealed using the mouse mAb DO-1 (N-terminal region specific: N-t) or the mouse mAb 421 (C-terminal region specific: C-t).

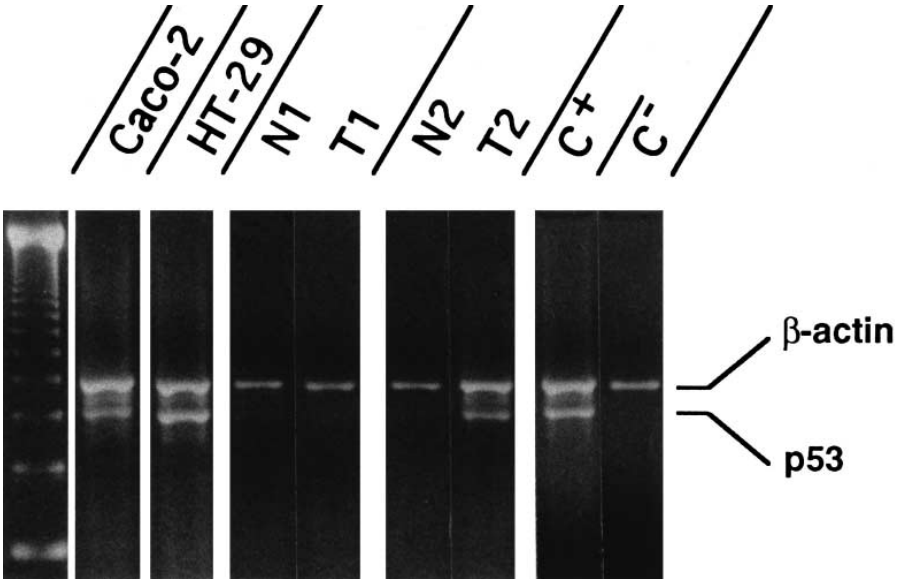

Fig. 6. Analysis of *p53* gene expression. The PCR products obtained from reverse-transcribed Caco-2 RNA were compared to those from HT-29 RNA, and two paired samples from human tissue resections for colon cancer (N1/T1 and N2/T2, N: non-tumoral mucosa; T: adenocarcinoma). Internal positive and negative controls of p53 RNA (C+: normal size p53 band from a colorectal carcinoma with increased accumulation of p53 transcript; C-: non-tumoral colonic mucosa with no detectable *p53* gene expression). The arrows indicate the migration positions of amplified RT-PCR products from *p53* and β-*actin* genes. First lane: molecular weight markers.

cally in non-tumoral colonic epithelial cells and stroma adjacent to sporadic colonic cancers [19], the status of the *p53* gene and its transcripts was therefore investigated in Caco-2 cells.

Direct sequencing of the PCR products corresponding to exons 5-9 of the *p53* gene revealed the presence of a stop codon in position 204 from exon 6. This cell line has lost the other allele of the *p53* gene. Fig. 6 shows the expression of the *p53* gene in Caco-2 cells. In comparative experiments, RT-PCR was also performed in the p53-positive HT-29 cell line and two human colon tumor samples. Expression was high in HT-29 cells (1.2 AU), lower in Caco-2 cells (0.35–0.39 AU) and much weaker or absent in a non-tumoral colonic mucosa, as compared to its corresponding adjacent primary tumor (0.03 and 0.36 AU in lanes N1 and T1, respectively). In contrast, the p53 transcripts were undetectable by RT-PCR in the other paired samples.

3.6. pRb2 status and interaction with SV40 LT

We next investigated the expression of the retinoblastoma-related phosphoprotein Rb2/p130, a negative regulator of proliferation and cell cycle progression [20], in SV40 LT-transfected human intestinal cell lines Caco-2 LT9 and CFI-3 [9], and parental or polyoma LT-transfected PC colonic epithelial cells (Fig. 7). By Western blot analysis, using the affinity-purified pAb directed against the spacer region of pRb2, we detected two autoradiographic bands at the expected molecular mass between 130 and 138 kDa, corresponding to different degrees of phosphorylation. In immunoprecipitates prepared from the SV40 LT-transfected CFI-3 and Caco-2 LT9 cells, we identified the 90 kDa antigen, indicating stable association between the viral oncoprotein and pRb2. In contrast, the polyoma LT antigen was not detected in pRb2 immunoprecipitates prepared from the PCPy/csrc cells transfected by the Polyoma early region (Fig. 7).

3.7. Expression of pRb2 in normal and transformed human colonic mucosa

Accumulation of Rb2/p130 protein was investigated in normal human colonic epithelial crypts and during the neoplastic progression of the human colonic mucosa. As shown in Fig. 8, pRb was undetectable in normal human colonic crypts (HC). The amount of pRb2 increased in tissue extracts prepared from Dukes' stages B1/B2, C2 or D, and their adjacent non-tumoral mucosa. The Rb2 protein was also detected in liver metastases of colonic cancers (M), but not in adjacent tissue (L).

4. Discussion

The gastrointestinal epithelia undergo a rapid and continuous renewal of their cell lineages from primitive stem cells localized in the gastric isthmus and intestinal crypts. In adults, these progenitors are mainly undifferentiated or committed as intermediary precursors of the epithelia. During fetal life in rats, proliferation and DNA synthesis is not restricted to this proliferating zone in the intestine and occurs along the emerging villi structures and stratified epithelium. In these conditions, oncogene-mediated immortalization of fetal and neonatal intestinal epithelial cells by the *SV40 LT* oncogene was previously associated with the emergence of poorly differentiated cell lines, with retention of some specific determinants of the digestive epithelia. This situation is also observed in conditionally immortalized intestinal epithelial cells from transgenic mice using the temperature-sensitive SV40 LT antigen under the control of an interferon γ-inducible H-2K^b promoter element directing the expression of the transgene in every cell [24]. These transgenic intestinal H-2K^b-tsA58 cells were found to be morphologically primitive with no morphological evidence of differentiation, and retained some specific biochemical markers of the normal digestive epithelia. Introduction of

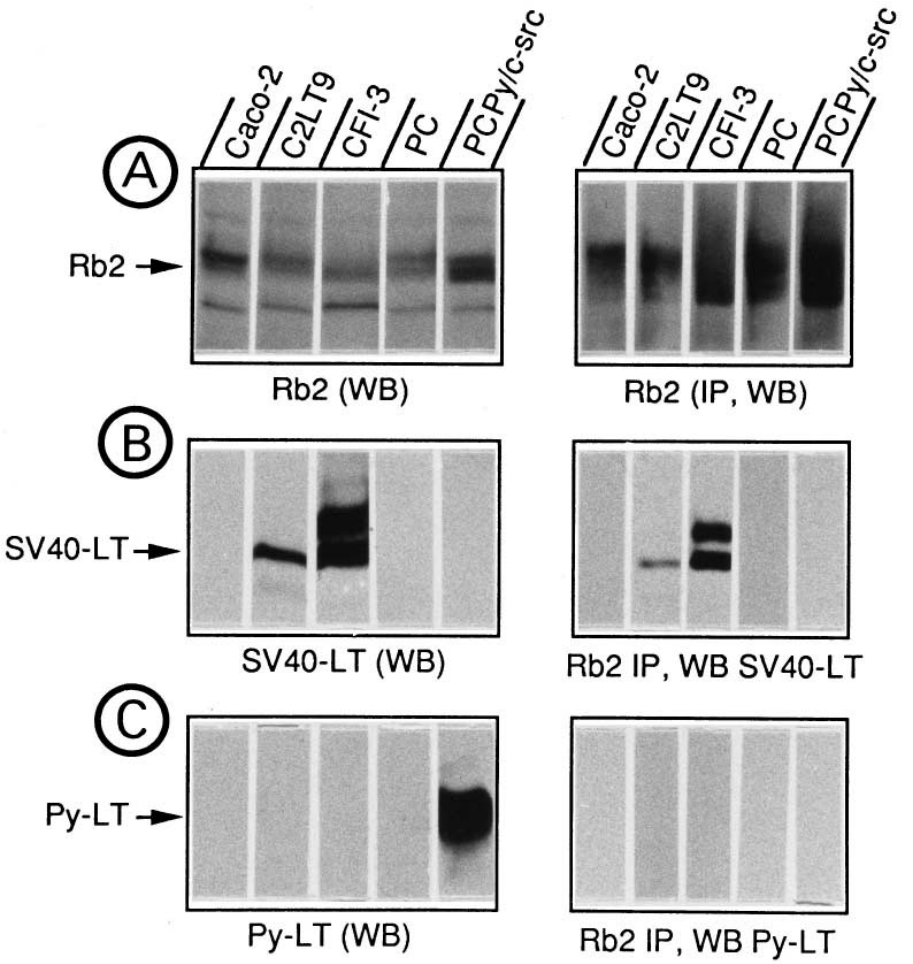

Fig. 7. pRb2 status and detection of the pRb2-SV40 LT complex. A: Immunoblot analysis of pRb2 in total cellular extracts (left) or in Rb2-immunoprecipitates (right). B: Detection of the SV40 LT antigen by Western blot (left), and pRb2–SV40 LT complexes in pRb2 immunoprecipitates prepared from the SV40 LT-transfected Caco-2 LT9 and CFI-3 cells (right). C: Immunoblot analysis of PyLT in total cellular extracts (left) or in pRb2-immunoprecipitates prepared from the parental or PyLT-transfected PC/AA cells.

thermolabile SV40 LT antigen under the SV40 early promoter in primary fetal intestinal epithelial cells also resulted in the expression of epithelial markers and induction of a more differentiated phenotype at the restrictive temperature [25]. Another elegant approach was to target the expression of SV40 LT in intestinal epithelial cells using tissue-specific regulatory sequences of the mouse intestinal *FABP* fatty acid protein gene [26,27]. In this transgenic I-FABP/SV40 LT antigen model, crypt-associated cells of the small intestine do not contain detectable levels of LT antigen since transgene expression is first detectable in enterocytes as they exit the crypts. The accumulation of the SV40 LT antigen in enterocytes persisted during migration along the crypt–villus axis, with no apparent effect on enterocytic differentiation markers, such as FABP, alkaline phosphatase, and morphology by light microscopic studies. However, there was no information on the ultrastructural organization and polarization of the LT antigen-expressing epithelial cells of the villi. When isolated from the I-FABP/SV40 LT transgenic intestine, epithelial cells did not produce any tumor in nude mice. Attempts to establish a permanent cell line from the same material were unsuccessful (J.I. Gordon, personal communication). In vivo, this transgenic intestine showed very mild dysplasia illustrated by bifurcated villi structures emerging from the crypts [27]. Establishment of transgenic animals using intestine-specific promoters already expressed in the proliferating crypt cells, such as the lactase-phlorizin hydrolase or OCT1 type transporters [28,29], or in the proliferative part of the colonic crypts, such as the *MSH2* gene [30], will allow precise evaluation of the consequences of SV40 LT expression on the proliferation and differentiation of the immature stem cells.

In this study, we have shown that expression of the *SV40 LT* oncogene in the human Caco-2 cell line resulted in minor changes in cell proliferation, tumor growth and differentiation, as judged by phase-contrast microscopy, ultrastructural organization or polarization, and expression of digestive enzymes. In contrast, we have previously shown that transformation of Caco-2 cells after transfer of the *ras* and *polyoma middle-T* oncogenes not only promoted cell proliferation and tumor growth but also impaired enterocytic differentiation [18,31,32]. Our data therefore support the hypothesis that terminal differentiation of the Caco-2 enterocytes is compatible with the expression of SV40 LT. One potential explanation for these results invokes the possibility that *SV40 LT* is a repressor of the differentiation program when this oncogene is expressed in undifferentiated proliferating intestinal epithelial

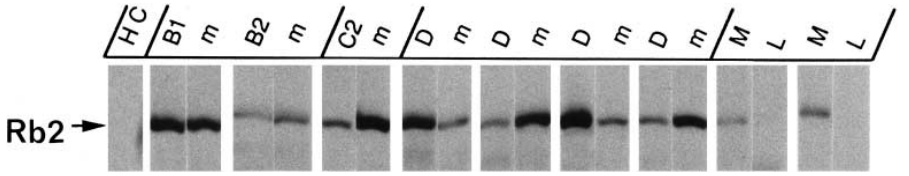

Fig. 8. pRb2 status in human colonic tumors. Protein samples (100 μg) were analyzed by Western blotting using the pAb against the C-terminal region of pRb2. Normal human colonic crypts: HC; Dukes' stages B1, B2, C2 and D; liver metastasis: M. The corresponding adjacent non-neoplastic tissue (colonic mucosa or liver) was analyzed for each paired resection from the same patient.

cells, and has permissive action in digestive cells that are already differentiated. Alternatively, it is also possible that the Caco-2 cell genotype exerts a dominant negative effect on the function of the *SV40 LT* oncogene.

In favor of this hypothesis, we first demonstrate here that the *p53* gene in Caco-2 cells has deleted and mutated alleles and no detectable accumulation of the corresponding protein. The stop codon in position 204 of the *p53* gene argues for a defective p53 protein at two levels. This putative truncated p53 protein in Caco-2 cells eliminates the possibility of the interactions with DNA binding sites or SV40 LT antigen in the domains located in the central part of the molecule (amino acids 100–293) and nuclear localization and oligomerization domains located in the C-terminal part of p53, that are required for the assembly of p53 tetramers. Consequently, this putative mutant form of p53 cannot activate transcription or exert its anti-oncogenic activity against LT antigen. Therefore, the proliferation/differentiation balance is not under the control of the tumor-suppressor gene *p53* in Caco-2 cells, suggesting that the wild-type p53 protein is not necessary for the expression of the enterocyte phenotype. Several authors have proposed that alterations in *p53* gene expression are associated with various cancers, such as those of the colon. Allelic deletion and p53 point mutation or protein accumulation rarely occur in preneoplastic lesions such as aberrant crypt foci of the colon or adenomatous colonic mucosa [1]. This finding supports the hypothesis that *p53* gene mutation or overexpression contributes to progression of colorectal carcinoma and is associated with short survival [33]. In contrast, p53 point mutations are found in the earliest recognized dysplastic lesions emerging from ulcerative colitis [34]. Recent data suggest that p53 controls cell cycle arrest at the G1/S transition in response to DNA damage [35], implicating p53 in negative regulation of cell proliferation. In this connection, our results provide evidence that Caco-2 cells are regulated by autocrine/intracrine mitogenic factors since parental and SV40 LT-transfected cell lines are growing in serum- and glutamine-free conditions. In the gut, glutamine functions as an essential nutrient maintaining intestinal structure, proliferation responses to growth factors such as EGF, and regulating expression of differentiation and adhesion markers in intestinal epithelial cells in culture [21–23]. Previous studies also indicated that human colon tumors and their derived cell lines express a variety of peptide growth factors including EGF, TGF-α, amphiregulin, IGFs, bFGF, and gastrin, which may regulate cell mitogenesis and growth at the autocrine level [36–40]. For example, the conditioned medium from Caco-2 cell cultures contained approximately 5–15 ng/ml immunoreactive IGF-II after 24 h of culture [41]. This IGF-II concentration produced half-maximal displacement of [^{125}I]IGF-II binding in the same system.

Secondly, the absence of functional p53 in Caco-2 cells is associated with high expression of the RB1 transcripts and abundance of the Rb1/Rb2 proteins in both parental and transfected cells. This may account for the negative effects of the p53 protein on *RB1* gene promoter activity, as previously evidenced in other systems [42,43]. High levels of pRb1-LT–p53 complex are also observed in SV40-transfected cells [44], suggesting that this trimolecular assembly may play specific roles in the transformation process induced by the viral oncogene. Cooperation between p53 and pRb1 in the control of apoptosis and predisposition to malignancy has been proposed from RB1 +/−, p53 −/− transgenic mice [45]. In contrast, normal human colonic crypts accumulated low levels of unphosphorylated Rb1 protein that is active in growth suppression and is a feature of the G1 phase of the cell cycle.

At the molecular level, the SV40 LT antigen was found in the pRb1 or Rb2 immunoprecipitates that we prepared from Caco-2 LT9 cells, resulting in the accumulation and persistence of hyperphosphorylated forms of pRb1 in post-confluent cells. The interactions between pRb1 and the SV40 LT antigen involves the 'Rb pocket' that can bind E1A of adenovirus and human papillomavirus type 16 E7 proteins [46]. Besides binding these viral oncoproteins, the hypophosphorylated forms of pRb1 also bind and allow the sequestration of the cellular protein E2F. The transcription factor E2F up-regulates the expression of proteins known to participate in cell growth regulation, such as myc, dihydrofolate reductase and EGF receptors [47]. Accordingly, hypophosphorylated pRb1 is almost completely absent in exponentially growing cells. After the acquisition of confluence and terminal differentiation, the three hyperphosphorylated forms of pRb1 are progressively converted into hypophosphorylated pRb1. This conversion is associated with down-regulation of the *c-myc* gene in parental and transfected Caco-2 LT9 cells (data not shown), and no further increase in cell number in 10 days post-confluent Caco-2 monolayers. The tumor-suppressors pRb1 and Rb2 may therefore contribute to the genetic and molecular alterations observed during the neoplastic progression of human colonic mucosa. Further analysis of Caco-2 cells expressing variable levels of p53 and pRb1/Rb2 by constitutive or inducible expression of the corresponding sense or antisense transcripts will be useful to evaluate the specific roles of these proteins in the proliferation and differentiation of human enterocytes.

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